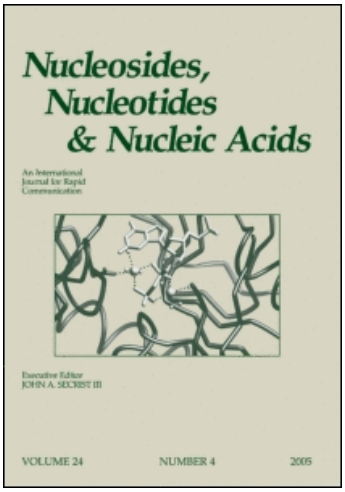

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The Mechanism of Dna Repair by Uracil-Dna Glycosylase: Studies Using Nucleotide Analogues

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THE MECHANISM OF DNA REPAIR BY URACIL-DNA GLYCOSYLASE: STUDIES USING NUCLEOTIDE ANALOGUES†

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ABSTRACT: 2',4'-Dideoxy-4'-methyleneuridine incorporated into oligodeoxynucleotides forms regular B-DNA duplexes as shown by T_m and CD measurements. Such oligomers are not cleaved by the DNA repair enzyme, UDG, which cleaves the glycosylic bond in dU but not in dT nor in dC nucleosides in single stranded and double stranded DNA. Differential binding of oligomers containing carbadU, 4'-thiodU, and dU residues to wild type and mutant UDG proteins identify an essential role for the furanose 4'-oxygen in recognition and cleavage of dU residues in DNA.

The repair of damaged DNA is essential for life. The deamination of deoxycytidine to deoxyuridine occurs spontaneously and is also subject to catalysis by UV and bisulphite. It generates a promutagenic U:G mismatch which, if not repaired, leads to a C–T transition mutation in the next round of DNA synthesis.[1,2] The enzyme Uracil-DNA Glycosylase (UDG) identifies the RNA-base uracil in a DNA duplex and hydrolyses the glycosylic linkage between C-1' of the deoxyribose and N-1 of uracil with a discrimination of not less than 10^7 against dT and dC residues.[3,4,5] This creates

† Dedicated to the memory of Sasha Krayevsky, good friend and fine colleague.

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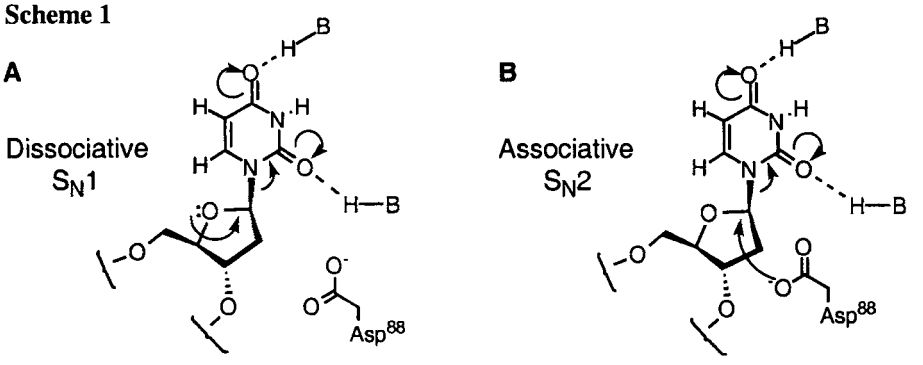
an abasic site which is subsequently further degraded by an AP endonuclease and deoxyphosphodiesterase.[6] It is estimated that there are some 100-500 of these repair events per cell per day in man.[7]

Crystal structures of the UDG from human[8] and from HSV-1[9] have been described and recently the flipping out and excision of a uracil base from the major groove of a DNA duplex bound to human UDG has been analysed in an X-ray structure of a product complex.[10] Roles for catalytic Asp64 and His187 in the *E. coli* enzyme have been proposed from NMR and X-ray crystallographic studies on the bacterial enzyme and its complex with uracil.[11,12] However, the mechanism of this enzyme-catalysed hydrolysis of the dU glycosylic bond remains but poorly understood.

Mechanistic studies on other enzymes that catalyse glycosyl transfer reactions[13,14] would suggest an S_N1-like, dissociative mechanism for the hydrolysis process for deoxyuridine,[10] in which hydrogen bonding to the uracil base at the C^2 and C^4-carbonyl oxygens of the pyrimidine is coupled to dissociation of the C1'-N glycosylic supported by the anomeric effect.[14] This would lead to an oxocarbenium-ion with cationic charge stabilised between C-1' and O-4' of the deoxyribose residue and possibly further stabilised by coulombic interaction with the proximate Asp88 anion or solvation in the active site (Scheme 1A). The alternative, associative process (Scheme 1B) invokes the nucleophilic participation of an oxyanion, either the essential Asp88 or a water molecule activated by that residue.[9]

Scheme 1



To support mechanistic studies on the hydrolyses of dU from DNA by the enzyme UDG, we seek to generate substrate analogues capable of discriminating between S_N2 and S_N1-like mechanisms. One solution to this problem is the synthesis of 2',4'-dideoxy-4'-methyleneuridine (carba-dU) and its incorporation into DNA oligomer

duplexes. Carbocyclic nucleosides have been used, *inter alia*, in enzyme studies on adenosine deaminase[15] and adenosine kinase.[16] In the present case for UDG, the replacement of the furanose oxygen by methylene would clearly inhibit the dissociative S_N1, or S_N1-like, process while its effect on an associative S_N2 process is less predictable. How such a substrate analogue interacts with WT and mutant enzymes should lead to a better understanding of the cleavage process and potentially of the basis of its prodigious selectivity.

Materials and Methods

NMR spectra were run on a Bruker AMX 250 instrument operating at 250.13 MHz for protons, 101.26 MHz for phosphorus, and 62.90 MHz for carbon. The construction, expression and purification of recombinant mutants of HSV1 UDG (D88N and H210N respectively) have been described previously.[17]

Synthesis of 5'-*O*-dimethoxytrityl-carba-2'-deoxyuridine CarbadU[18] (260 mg, 1.16 mmol) was co-evaporated with dry pyridine (3x), dissolved in dry pyridine/ dichloromethane 1:1 (12 ml) and 4,4'-dimethoxytrityl chloride (432 mg, 1.28 mmol) and triethylamine (20 ml) added and the mixture left 2 d at 4 °C. Methanol (10 ml) was added and the reaction mixture stirred 5 min at rt. Solvent was removed *in vacuo* and the residual oil dissolved in ethyl acetate and washed with sat $NaHCO_3$. The organic layer was dried over Na_2SO_4 and evaporated. Flash chromatography on silica gel, eluting with a toluene-ethyl acetate/methanol (9:1) gradient afforded a colourless foam of **3** (73 %, 450 mg). RF: 0.47 (toluene-ethyl acetate/methanol 9:1). ^{1}H NMR ($CDCl_3$): δ = 1.90-2.10 (5 H, m, H2',2",4',6',6"), 3.60 (H, dd, H5'), 3.55 (1H, dd, H5"), 3.78 (6H, s, 2 x OCH_3), 4.39 (1H, dd, H3'), 5.07 (1H, ddd, H1'), 5.28 (1H, d, H5), 6.80 (4H, m, *o* to OMe), 7.45 (1H, d, H6'), 7.2-7.6 (9H, m, ArH), 8.5 (1H, b, OH), HRMS (CI+): calcd for $C_{31}H_{32}N_2O_6$ 528.22604; found 528.22487

5'-*O*-Dimethoxytrityl-3'-*O*-(cyanoethoxy-diisopropylaminophosphane)-carba-2'-deoxyuridine. The above product (246 mg, 0.45 mmol) was dissolved in dry CH_3CN (5 ml) then cyanoethoxy-bis(diisopropyl)aminophosphane (272 mg, 0.9 mmol) and 1H-tetrazole (16 mg, 0.23 mmol) were added. The solution was stirred under nitrogen for 16 h then the mixture diluted with CH_2Cl_2 (20 ml) and washed with sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* and the residue subjected to flash chromatography on silica gel, eluting with a toluene:ethyl acetate (2:1) gradient with 0.2 % triethylamine to give **4** (82 %, 268 mg) as a colourless foam. RF: 0.52, 0.64 (toluene/EtOAc 1:1) ^{1}H NMR ($CDCl_3$): δ = 1.0-1.3 (12 H, m, 2 x $CH(CH_3)_2$), 2.0-2.7 (7H, m, H2',H2", H4', H6', H6", OCH_2CH_2CN), 3.1-3.7 (6H, m, H5', H5", OCH_2CH_2CN, 2 x $CH(CH_3)_2$), 3.75 (6H, 2s, 2 x OCH_3), 4.4 (1H, m, H3'),

5.21 (1 H, m, H1'), 5.30 (1H, d, H5), 6.78 (4 H, m, 4*o* to OMe), 7.1-7.7 (10 H, m, 9 x ArH, H6). ^{31}P NMR ($CDCl_3$): δ = 148.74, 148.30.

Cleavage studies - Oligodeoxynucleotide synthesis

Carba-dU-modified oligonucleotides were synthesised using an Applied Biosystems Inc. model 294 DNA synthesiser with conventional phosphoramidite chemistry and standard protocols for DNA synthesis. A solution of carbadU phosphoroamidite (**4**, 0.1 M) in acetonitrile:dichloromethane 5:1 was applied to the synthesiser. CarbadU units were introduced using an increased waiting time of 3 min. The oligonucleotide was cleaved from the CPG support then purified by preparative gel-electrophoresis on a 15 % denaturating polyacrylamide-bisacrylamide gel. Sequences used for the cleavage studies included: d(TGC-CTA-AU*G-AGT-GAG), d(TGC-CTA-AUG-AGT-GAG), and d(TGC-CTA-ATG-AGT-GAG).

These oligomers were 5'-end labelled using polynucleotide kinase (NEB, Boston)[19] and γ-^{32}P-ATP (Amersham). Each labelling reaction was allowed to proceed for 30 min after which unincorporated nucleotide was removed by gel filtration.[17] Unless otherwise stated in legends, the final concentration of oligonucleotides used was 25 nM. UDG was buffer exchanged by dialysis to Tris/HCl buffer pH 8.2 (10 mM Tris, 5 mM EDTA, 0.02 % sodium azide). The enzyme was concentrated with Centricon-10 concentrator (Amicon Inc) to a stock concentration of 10 pmol/ml (5μM), according to manufacturer's instructions and 10, 100, 1000 and 5000 fold dilutions were prepared.

Labelled oligonucleotides (5 μl, 0.1 μM), Tris buffer pH 7.8 (13 μl, 50 mM Tris, 10 mM EDTA, 100 mM NaCl, 0.1 mg/ml BSA) and UDG (2 μl, 500 M, 50 nM, 5 M, 1 nM) were incubated at 37 °C for 1 h. NaOH (30 ml 0.1 M) was added and the mixture heated to 95 °C for 10 min. TBE buffer (50 μl 1x) was added and the reaction mixture subjected to a 15 % denaturing polyacrylamide/bisacrylamide gel and analysed by gel electrophoresis.

Synthesis and purification of oligonucleotides for BIAcore binding studies

The oligonucleotide sequence used for these experiments was 5'-Biotin-d(CCGAATCAGTTCACTTCU*AGCCGAGGTATTTAGCC), in which U* represents carba-dU. The non-biotinylated oligos were 5'-GGCTAAATACCTCGGCTRGAAGTGAACTGATTCGG where R is either A or G to form the duplexes dsU/A and dsU/G respectively with the biotinylated strand ssU. Oligonucleotide synthesis was performed on an Applied Biosystems 394 DNA synthesiser on the 0.2 mmole scale using cyanoethyl phosphoramidite chemistry. Standard DNA synthesis reagents and cyanoethyl-phosphoramidite monomers were obtained from Applied Biosystems Ltd. The biotin phosphoramidite was obtained from Cruachem Ltd and was used as a 0.15 M solution in anhydrous acetonitrile with the

coupling time extended to 3 min. Stepwise coupling efficiencies were measured on the synthesiser by trityl analysis and all monomers coupled at greater than 98 %. Oligonucleotides were deprotected in concentrated aqueous ammonia for 8 h at 55 °C.

HPLC purification was carried out on a Gilson model 306 HPLC system using a Brownlee Aquapore Octyl reverse phase column (10 mm x 250 mm) with a flow rate of 3 ml/min and a gradient of 0 % to 75 % buffer B over a period of 30 min. (Buffer A: 0.1 M triethylammonium acetate, buffer B: 0.1 M triethylammonium acetate with 25 % acetonitrile). In the case of oligonucleotides labelled with biotin, the desired product was the major peak (final peak to elute from the column). After HPLC purification the major product was evaporated to dryness and desalted using a Pharmacia NAP 10 column (Sephadex G25), according to manufacturer's instructions.

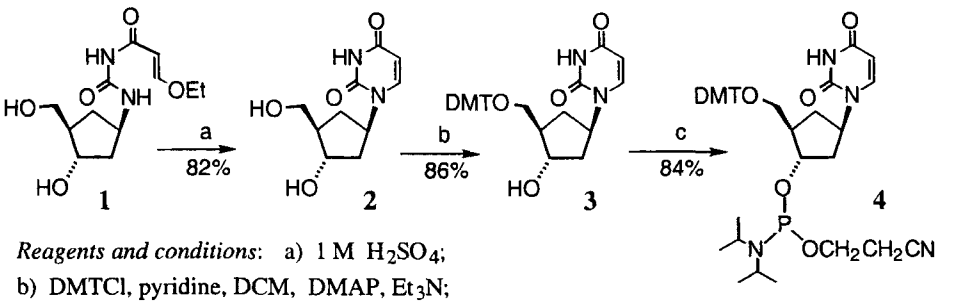
Experimental assay procedure using Surface Plasmon Resonance The operation of the BIAcore biosensor and its use in analysing protein-DNA interactions have been described before.[17] All interactions were analysed in binding buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005 % Tween-20) at a constant flow rate of 5 µl/min and at a constant temperature of 25 °C. Biotinylated oligonucleotides were injected over a streptavidin-coated sensor chip (SA5, Pharmacia Biosensor) until a suitable signal level was achieved (see Results section). For the formation of double-stranded oligonucleotides, non-biotinylated DNA was injected until no more increase in binding to the immobilised single-stranded DNA could be observed. Bound protein was eluted from the DNA by a short pulse (5 µl) of 0.05 % SDS. This regeneration procedure did not alter to any measurable extent the ability of the immobilised DNA to bind protein in subsequent cycles. Data analysis was performed using the evaluation software supplied with the instrument. In order to eliminate small "bulk" refractive change differences at the beginning and end of each injection (resulting from small differences in buffer composition of the stock protein solutions) a control sensorgram obtained over a non-binding surface was subtracted for each protein injection.

Results

(+)-(1*R*,2*S*,4*R*)-4-Amino-2-hydroxy-1-hydroxymethylcyclopentane[20] was condensed with 3-ethoxycrotonyl isocyanate to give carba-dU **1** which was cyclised by heating with acid to give 2',4'-dideoxy-4'-methyleneuridine **2** (Scheme 2). To support standard phosphoramidite chemistry for DNA synthesis, the 5'-hydroxyl group of **2** was dimethoxytritylated to afford **3** whose 3'-OH function was phosphitylated with 2-cyanoethoxy-bis(diisopropyl)amino phosphane[21] with 1*H*-tetrazole catalyses in the usual manner and in moderate yield to give **4** (Scheme 2). The resulting phosphoramidite **4** was directly incorporated into DNA by automated synthesis

following slightly modified protocols in a "trityl-on" synthesis. Two oligodeoxynucleotides were thus prepared having a central modified base 5'-d(TGC-CTA-AU*G-AGT-GAG) **5** and 5'-d(GAC-GTG-GGU*-GGC-TTG-ACG) **6**, in which U* represents carba-dU. Additionally, an oligodeoxynucleotide 5'-Biotin-d(CCG-AAT-CAG-TTC-ACT-TCU*-AGC-CGA-GGT-ATT-TAG-CC) **7** was prepared for use in surface plasmon resonance studies using BIAcore with complementary oligodeoxynucleotides described previously.[17] The reaction products were purified by reversed phase chromatography and the resulting oligonucleotides were 5'-*O* deprotected in the usual way with 80 % acetic acid.

Scheme 2



Reagents and conditions: a) 1 M H_2SO_4;
b) DMTCl, pyridine, DCM, DMAP, Et_3N;
c) $[(Pr^i)_2N]_2POCH_2CH_2CN$, 1*H*-tetrazole / CH_3CN.

To show that the carba-dU modification does not cause any loss of helix stability, T_m measurements were made on three synthetic pentadecadeoxynucleotides (Table 1). These show that the change from a central dT:dA base-pair to either a dU:dA or a carba-dU:dA base-pair results in a lowering of the T_m by 2 °C. Thus, all three duplexes are fully intact under the conditions used in the subsequent enzyme hydrolysis studies since a base-mismatch would lead to a lowering[22] in T_m of some 6-7 °C. The CD spectrum of the dodecamer containing the carbadU residue (Table 1, entry 3) showed no divergence from that of a normal B-duplex (data not shown).

To establish whether the deoxyuridine nucleoside analogue is resistant to hydrolysis, the three oligodeoxynucleotides were 5'-^{32}P-end-labelled and incubated for 1 h with the UDG. After treatment with 0.1 M NaOH for strand scission at abasic sites generated by glycohydrolase action, the products were analysed on a high percentage denaturating polyacrylamide gel. When the oligodeoxynucleotide 5'-d(TGC-CTA-AUG-AGT-GAG) **8** containing an unmodified deoxyuridine residue was incubated with varying amounts of the enzyme UDG and treated with 0.1 M NaOH, concentration-dependent cleavage product was observed (Fig. 1, panel II). In contrast oligomers **5** and **6** containing the

Table 1 Melting temperatures for dU:dA and carba-dU:dA containing duplexes

Duplex Sequence	T_m value
5'-d(TGC-CTA-ATG-AGT-GAG)-3' 3'-d(ACG-GAT-TAC-TCA-CTC)-5'	57.1 ± 0.5 °C
5'-d(TGC-CTA-AUG-AGT-GAG)-3' 3'-d(ACG-GAT-TAC-TCA-CTC)-5'	55.0 ± 0.5 °C
5'-d(TGC-CTA-AU*G-AGT-GAG)-3' 3'-d(ACG-GAT-TAC-TCA-CTC)-5'	55.0 ± 0.5 °C

Transitions measured at 260 nm in tris-buffer/HCl pH 8, 0.1 M [Na^+], U* = carba dU. Binding properties of the carba-dU-modified oligonucleotides were measured using an PTP-1 Peltier Element (Perkin Elmer) with an UV-Lambda 2 spectrometer (Perkin Elmer). Both oligonucleotide pentadecamer duplexes containing dU:dA and carba-dU:dA as modified base-pairs show a 2 °C depression in T_m compared to the parent dT:dA duplex.

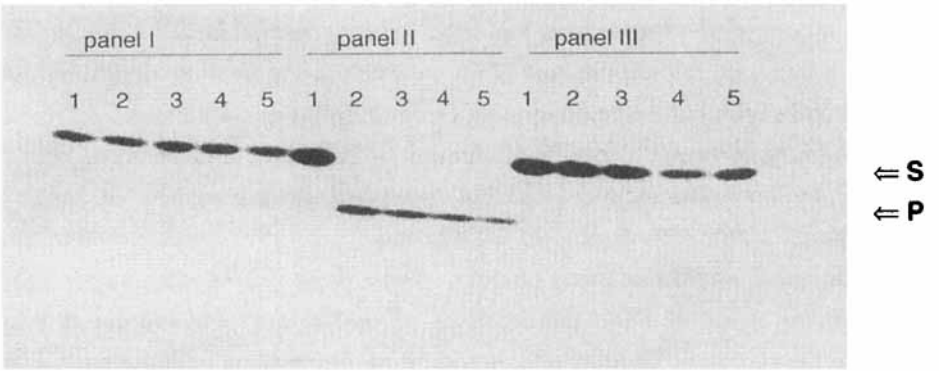


FIG. 1 Comparison of uracil removal from single stranded DNA substrates. 5'-^{32}P-Labelled d(TGC-CTA-AU*G-AGT-GAG) (Panel I), 5'-^{32}P-labelled d(TGC-CTA-AUG-AGT-GAG) (Panel II), 5'-^{32}P-labelled d(TGC-CTA-ATG-AGT-GAG) (Panel III) were incubated with different amounts of UDG in tris-buffer for 1 h at 37 °C followed by 0.1 M NaOH. Oligonucleotide concentration 25 nM; UDG concentrations all panels: lane 1, no protein; lane 2, 50 nM; lane 3, 5 nM; lane 4, 500 pM, lane 5, 100 pM. (Product (**P**) - lower tracks; substrate (**S**) - upper tracks).

carba-dU migrated unchanged even after incubation with a two-fold molar excess of enzyme (Fig. 1, panel I). Similarly, the dT-containing oligomer **9** also fully resisted enzymic degradation (Fig. 1, panel III).[¶]

Gel-shift studies on the carba-dU oligomers with UDG suggested that these oligos have a somewhat reduced affinity for the enzyme. We therefore studied the binding of both single strand and double strand oligos to UDG by surface plasmon resonance using a BIAcore 2000 instrument.[17] The affinity of WT enzyme for the oligomer containing the carba-dU residue, 5'-d(CCG-AAT-CAG-TTC-ACT-TCU*-AGC-CGA-GGT-ATT-TAG-CC), gave a K_d value 10^{-6} M, towards the upper limit of SPR analysis. By contrast, the H210N mutant of UDG, shown to be catalytically inactive for native substrate[15] and closely related to the double mutant UDG employed in crystallographic studies on a complex of the human enzyme with products,[10] and the inactive D88N mutant display no changes in either the association or dissociation rates for carbadU DNA **7** from those published[15] for dU-DNA, giving values of K_d 10^{-8} M. The results obtained for dsDNA duplexes were indistinguishable from those measured for ssDNA.

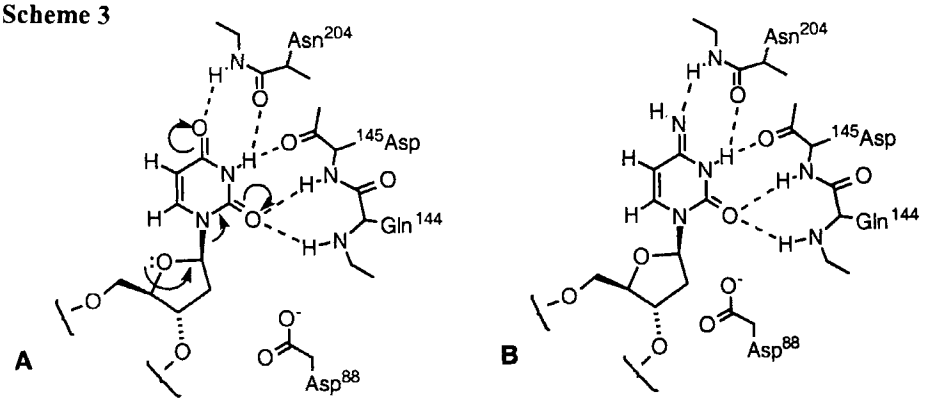
Discussion

Taken together, these results establish that carbocyclicdeoxyuridine can be introduced into a DNA duplex with little loss of helix stability. The small lowering of T_m is indistinguishable from that seen for the dT–dU substitution (Table 1). The CD spectrum shows no divergence from that expected for a normal B–DNA duplex. This result is in line with calculations and NMR studies[23] and with x-ray crystallographic data[24] on N-S conformational equilibria for carbocyclic nucleosides.

The resulting oligomer is not itself cleaved by the UDG. By contrast, both the regular deoxyribouridine and the 4'-thio-2',4'-dideoxyribouridine residues are subject to glycohydrolase action (Savva, R., Pearl, L.H., and Walker, R.T. unpublished results), albeit with some retardation for 4'-thiodU. Thus, these results offer support for a mechanism of action of UDG that involves an S_N1 or S_N1-like process in which scission of the glycosylic bond involves transient formation of an oxocarbenium cation species at C-1' of the deoxyribose residue, essentially stabilised by O-4' (Scheme 3 B). The slower reaction of the 4'-thio-dU is consistent with a reduced anomeric contribution from sulfur relative to oxygen in the furanose ring.[14] It is fully compatible with a role for the carboxylate residue (Asp88 in the viral enzyme and Asp145 in the human enzyme) as providing some measure of charge stabilisation of a transient oxocarbenium ion.

[¶] Preliminary MS analysis of the carba-dU strand supports this conclusion.

Scheme 3



The replacement of the 4'-oxygen function by methylene leads to a 100-fold reduction in affinity of the WT enzyme for the carba-dU-containing oligomer. While this affinity remains adequate for the oligomer 5'-d(TGC-CTA-AU*G-AGT-GAG) to bind to WT UDG under the conditions employed for cleavage analysis (Figure 1) and support the conclusion that it is a stable substrate analogue, the unattenuated affinity of the D88N and H210N mutant proteins for the carba-dU containing oligomer is unexpected. It appears unlikely to result from a retardation of the rate of conformational exchange of pseudorotamers.[23] One possible explanation is that UDG has evolved to recognise the transition state corresponding to substrate cleavage, which enables the uracil residue to interact more effectively with amino acid residues in the affinity pocket identified in the X-ray structure of the product complex.[10] An alternative is that the slow conformational change that is involved in the discrimination between weak non-specific binding of UDG to dsDNA and tight binding to substrate[11,12] is retarded further in the case of the carbadU substrate to an extent that makes the BIAcore experiment unable to measure the true "on-rate" for binding.

CarbadU is recognised in the active site, as shown by the same high affinity of mutant UDG for both dU-DNA and carbadU-DNA. However, WT-UDG does not ordinarily reside on a dU-DNA substrate, as shown by our BIAcore data with WT-UDG for both dU-DNA and carbadU-DNA. It thus appears that UDG is primed for immediate scission of the glycosylic bond as soon as the active site pocket is occupied, which is itself a slow and highly selective process. When a base appears in the UDG active site and fits into the detailed hydrogen bonding network, it is cleaved immediately. Any structural modification to the deoxyuridine that disfavours the conformation required for such binding will therefore reduce the apparent affinity of the residue for UDG, and may do so differentially with respect to the mutant enzymes.

Taken together, these considerations appear to imply that UDG binds its specific substrate in a Michaelis-Menten complex of high energy. It seems likely that dU interacts with residues in the UDG active site involving a high-energy distortion that can only be achieved through the presence of the 4'-oxygen, particularly involving electron donation to C1' by a classical anomeric effect not available for the carbadU analogue.[27] This conclusion clearly directs our further studies to the use of nucleoside analogues which retain the furanose oxygen and achieve stability, *e.g.* through a C-nucleoside functionality as in pseudo-deoxyuridine.

The present studies may also bear on the question whether the 10^7 discrimination of UDG against cleavage of the glycosylic bond of deoxycytidine is determined by molecular recognition in binding dU while rejecting[8] dC or is a consequence of kinetic, *i.e.* transition state, discrimination. The observation that the mutant N204D in the human enzyme does in fact cleave dC residues,[25] albeit at a reduced rate, has been interpreted in favour of discrimination through binding. However, it would appear that the disfavoured imino-tautomer of cytosine could be accommodated equally well in the base-recognition site (Scheme 3 B) but would be a *poorer leaving group* in a dissociative process. Its protonation by Asp204 in the N204D mutant could well reverse that selectivity. This problem will be addressed by the use of further nucleoside analogues in future studies.

Conclusion

This investigation established that for glycohydrolases operating on DNA mismatches, the use of 2',4'-dideoxy-4'-methyleneribose is a useful probe for exploring the mechanism of base cleavage. Results using this probe indicate that the cleavage mechanism for UDG is likely dissociative in nature. The ribose modification provides an alternative to the 2'-deoxy-2'-fluoro-*arabino*furanosides, recently exemplified in studies on the DNA repair enzyme ANPG, an alkyl-*N*-purine DNA glycosylase.[26,27] Both of these sugar modifications would appear to lend themselves to studies based on crystal structure analysis.

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